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Reviewer Comments

In this paper authors examined apoptosis of FaDu cells following treatment with demethoxycurcumin for 24h. Nothing is new in the paper except the cell line, the paper has no translational importance and all the experiment is done in single cell line. None of the effect is studied at IC_{50} dose and long term effect of the drug is not included. There is no normal control, positive control and vehicle control. Induction of apoptosis and inhibition of NF-kB might be two independent mechanisms, there is no experimental evidence or discussion that can prove "DMC-induced FaDu cell apoptosis was mediated by the modulation of the NF-κB cellular signaling pathway".

Reply: First of all, we appreciate your valuable comments to improve the academic quality of the study. I agree with reviewer's comments. The anti-cancer effects of demethoxycurcumin (DMC) as a derivatives of curcumin have been reported in various types of cancers such as human cervical cancer (1), human epithelia ovarian cancer (2), cisplatin-resistant non-small lung cancer (3), prostate cancer (4), human breast cancer (5), and oral cancer (6). However, it is not yet reported the anti-cancer effect of human head and neck squamous cell carcinoma (HNSCC) in the field of oral and maxillofacial surgery. Hence, although the anti-cancer effects of DMC were very well studied in various cancer cells, but we think that it also might have an academic property associated with the anti-cancer effect and its cellular signaling pathways of DMC in HNSCC.

None of the effect is studied at IC_{50} dose and long term effect of the drug is not included.

In accordance with reviewer' comment, to determine the IC_{50} of DMC in the HNSCC, cell cytotoxicity using MTT assay were performed again in the HNSCC treated with 0, 1, 5, 20, 50, and 100 μM DMC for 24 h. As shown in the result of MTT assay (Figure 1), relative cell viabilities were measured by 101.14 ± 0.6%, 85.7 ± 0.6%, 68.2 ± 1.6%, 30.1 ± 2.3%, and 21.5 ± 3.2% in the in the HNSCC treated with 0, 1, 10, 20, 50, and 100 μM DMC, respectively, compared with untreated control (100 ± 0.2%). Based on these results, IC_{50} value is assessed by 35.5 μM DMC in HNSCC.

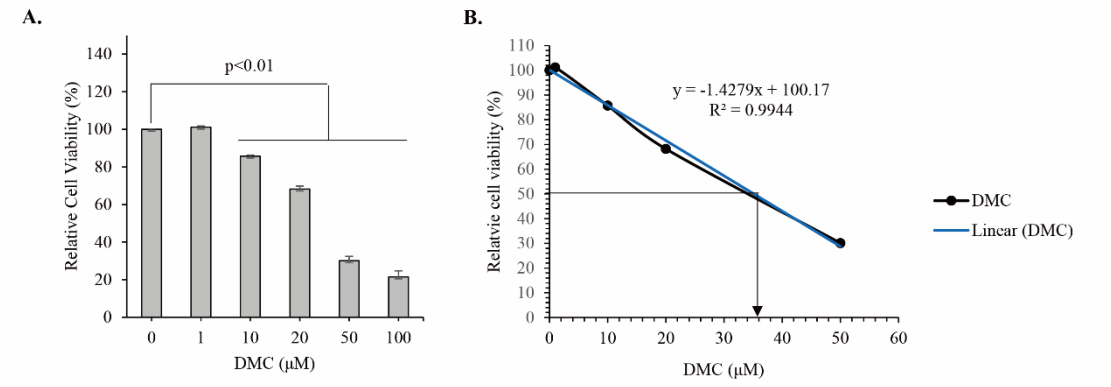

Figure 1. The cell viability and IC_{50} value of DMC in FaDu cells. FaDu cells were treated with 0, 1, 10,

20, 50 and 100 μM DMC for 24 h. Thereafter, MTT assay was performed to measure the relative cell viability of FaDu cells. IC_{50} value of DMC was estimated based on the result of MTT assay

As shown in the result of MTT assay, the relative cell viability was dramatically decreased in the FaDu cells treated with 50 μM DMC (30.1 ± 2.3%), compared with that of 20 μM DMC (68.2 ± 1.6%). Hence, the long term effect of DMC was not considered in FaDu cells. However, as the reviewer's comment, FaDu cells were treated with 0, 1, 10, 20, 50 and 100 μM DMC for 24, 48, and 72 h. Thereafter, MTT assay was performed as shown in Figure 2.

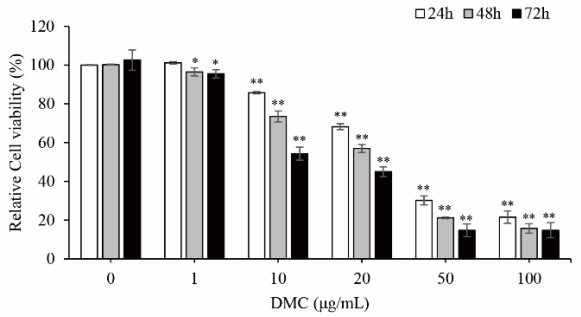

Figure 2. The cell viability of FaDu cells treated with DMC according to a time-dependent culture period. FaDu cells were treated with 0, 1, 10, 20, 50 and 100 μM DMC for 24, 48, and 72 h. Thereafter, MTT assay was performed to measure the relative cell viability of FaDu cells.

As shown in Figure 2, in the FaDu cells treated with over dosage of 50 μM DMC for 48 and 72 h, relative cell viabilities were almost 20% or less. To perform the western blot and other studies, the quantity of examining samples such as total proteins extracted from cells are so less. Therefore, we determined the anti-cancer properties of FaDu cells treated with10 and 20 μM DMC for 24 h. Please understand it. Hence, there were revised the part of materials & methods, results, Figure 2 and its figure legend in revised manuscript.

There is no normal control, positive control and vehicle control.

5% DMSO, an organic solvent to resuspend many materials, can be used successfully for some cells. Moreover, although 0.5% DMSO is recommended as the final concentration for cell culture without cytotoxicity, 1% DMSO dose not cause any toxicity to some cells (Ref. https://www.lifetein.com/chat/932729-DMSO-usage-in-cell-culture).

According to data sheet supplied from manufacture (Sigma-Aldrich, St. Louis, MO, USA), the solubility of DMC (D7696, MW 338.35) is ≥ 10mg/mL in dimethyl sulfoxide (DMSO). 100 mM (3.3835mg/mL) DMC was prepared as a stock to use in present study. In present study, the final concentration of DMSO for the treatment of 100 μM DMC is 0.1%, which is considered to be safe for almost cells. Therefore, untreated control might be regarded as normal control and vehicle control.

In addition, this study is not a comparative study to compare other

chemotherapeutic reagents for the patient with head and necks squamous cell carcinoma. Hence, positive control was not used in present study. Please understand it.

Induction of apoptosis and inhibition of NF-kB might be two independent mechanisms, there is no experimental evidence or discussion that can prove "DMC-induced FaDu cell apoptosis was mediated by the modulation of the NF-κB cellular signaling pathway".

I agree with reviewer's comment. However, present study demonstrates DMC not only induce the apoptosis of FaDu cells through the death receptor mediated extrinsic and mitochondria dependent intrinsic apoptosis pathway, but also DMC suppress the nuclear translocation of NF-κB through the inhibition of its phosphorylation in FaDu cells. NF-κB is closely associated with oncogenesis through the regulation of gene expression associated with the development and progression of cancer such as proliferation, migration and apoptosis (7). Hence, aberrant or constitutive activation of NF-κB has been detected in many human malignancies.

Especially, NF-κB is closely associated with transcription of genes involved in the suppression of cell death by both death receptor mediated extrinsic and mitochondria dependent intrinsic apoptosis pathways. The phosphorylation of NF-κB may upregulate the expression of interfering proteins such as FLICE-like inhibitory protein (FLIP) that prevent caspase-8 recruitment to death-inducing signaling complex (7-9). Hence, the inhibition of NF-κB phosphorylation induces the death receptor mediated extrinsic apoptosis through the suppression of FLIP expression.

Furthermore, NF-κB induces the expression of anti-apoptotic factors such as the inhibitors of apoptosis (IAPs) and Bcl-2 family. IAPs such as c-IAP1, c-IAP2, and XIAP suppress apoptotic cell death through directing inhibition of caspases such as caspase-3 and caspase-9 (10-12). Bcl-2 family increase the mitochondrial outer membrane permeabilization (MOMP) through antagonize the function of pro-apoptotic factor such as Bad (13,14). These are indicating that the suppression of NF-κB phosphorylation is closely associated with the induction of apoptotic cell death that is mediated by the mitochondria dependent apoptosis pathway.

Changes in the text: we have modified our text as advised (see Page 4, line 21) as below

→ Thereafter, the cells were treated with 0, 1, 10, 20, 50, and 100 μM DMC for 24 h at 37 °C.

Changes in the text: we have modified our text as advised (see Page 9, line 8-12) as below

→ FaDu cells were treated with treated with 0, 1, 5, 20, 50, and 100 μM DMC for 24 h. Thereafter, MTT assay were performed to verify the viability and survival of FaDu cells. The results of the MTT assay showed that relative cell viabilities were measured by 101.14 ± 0.6%, 85.7 ± 0.6%, 68.2 ± 1.6%, 30.1 ± 2.3%, and 21.5 ± 3.2% in the in the HNSCC treated with 0, 1, 10, 20, 50, and 100 μM DMC, respectively, compared with untreated control (100 ± 0.2%),

Changes in the text: we have modified our text as advised (see Page 15, line 4-20) as below

→ Generally, NF-κB is closely associated with oncogenesis through the regulation of gene expression associated with the development and progression of cancer such as proliferation, migration and apoptosis (7). Hence, aberrant or constitutive activation of

NF-κB has been detected in many human malignancies (15-17). Especially, NF-κB is closely associated with transcription of genes involved in the suppression of cell death by both death receptor mediated extrinsic and mitochondria dependent intrinsic apoptosis pathways. The phosphorylation of NF-κB may upregulate the expression of interfering proteins such as FLICE-like inhibitory protein (FLIP) that prevent caspase-8 recruitment to death-inducing signaling complex (7-9). Hence, the inhibition of NF-κB phosphorylation induces the death receptor mediated extrinsic apoptosis through the suppression of FLIP expression. Furthermore, NF-κB induces the expression of anti-apoptotic factors such as the inhibitors of apoptosis (IAPs) and Bcl-2 family. IAPs such as c-IAP1, c-IAP2, and XIAP suppress apoptotic cell death through directing inhibition of caspases such as caspase-3 and caspase-9 (10-12). Bcl-2 family increase the mitochondrial outer membrane permeabilization (MOMP) through antagonize the function of pro-apoptotic factor such as Bad (13,14). These are indicating that the suppression of NF-κB phosphorylation is closely associated with the induction of apoptotic cell death that is mediated by the mitochondria dependent apoptosis pathway.

References

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Other comments:

1. Fig 2B: needs quantitation. Authors should provide lower magnification representing whole field. Length of scale bar is not clear.

Reply: According to reviewer's comment, Figure 2B was revised in the revised manuscript as below.

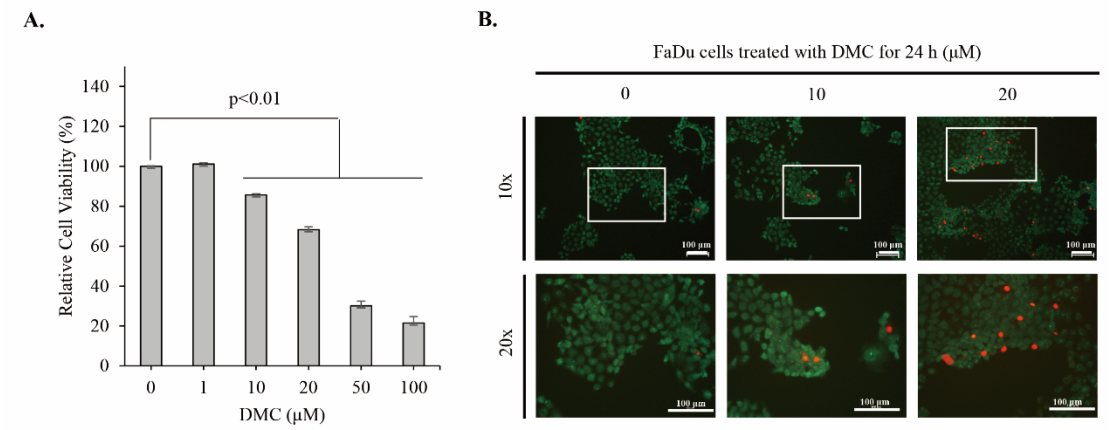


Figure 2 DMC decreased the viability and survival of FaDu cells. FaDu cells were treated with 1, 10, 20, 50, and 100 μM DMC for 24 h. Thereafter, MTT assay (A) was performed to measure the cell viability and survival of FaDu cells treated with DMC. To perform Live/Dead cell staining (B), FaDu cells were treated with 10 and 20 μM DMC for 24 h. Thereafter, live and dead cells were imaged using a fluorescence microscope (Eclipse TE2000; Nikon Instruments, Melville, NY, USA). (A) Cell viability was decreased by DMC in FaDu cells. (B) Cell survival was decreased by DMC in FaDu cells.

Changes in the text: we have modified our text as advised (see Page 23, line 5-11) as below

→ Figure 2 DMC decreased the viability and survival of FaDu cells. FaDu cells were treated with 1, 10, 20, 50, and 100 μM DMC for 24 h. Thereafter, MTT assay (A) was performed to measure the cell viability of FaDu cells treated with DMC. To perform Live/Dead cell staining (B), FaDu cells were treated with 10 and 20 μM DMC for 24 h. Thereafter, live and dead cells were imaged using a fluorescence microscope (Eclipse TE2000; Nikon Instruments, Melville, NY, USA). (A) Cell viability was decreased by DMC in FaDu cells. (B) Cell survival was decreased by DMC in FaDu cells.

2. No changes are seen in Fig 3A, B and C. Please revisit the data and provide lower magnification images showing whole field with magnified portion showing the changes by arrows.

Reply: According to reviewer's comment, Figure 3 was revised in the revised manuscript as below.

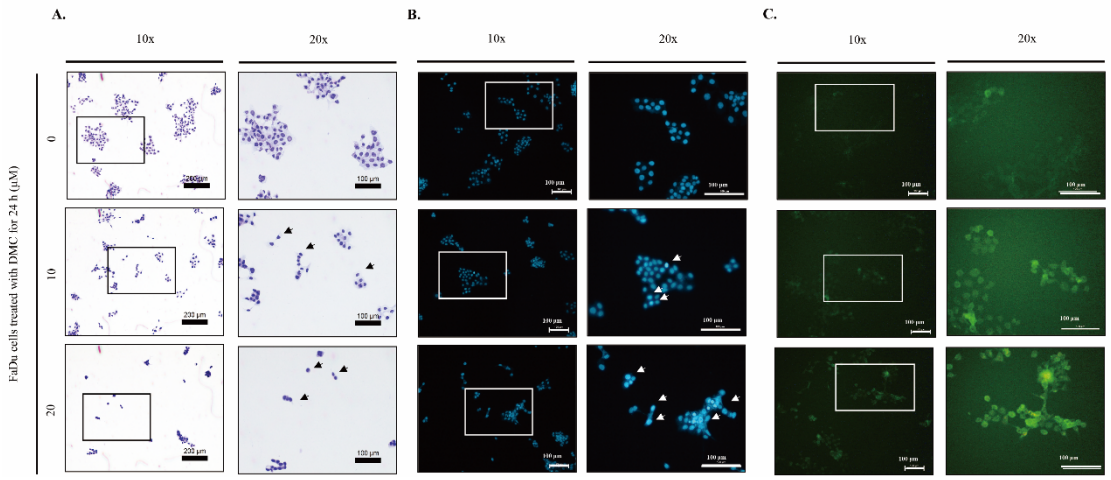


Figure 3 Apoptotic characteristics such as alteration of morphology, condensed nucleus, and caspase-3/-7 activity were observed in FaDu cells treated with DMC. Cultured FaDu cells were treated with 10 and 20 μM DMC for 24 h. Thereafter, H&E staining (A) and DAPI staining (B) to investigate the morphological alteration and nucleus condensation, respectively. In addition, to perform the caspase-3/-7 activity assay (C), FaDu cells were treated with 10 and 20 μM DMC for 24 h. Thereafter, cells were stained with the cell-permeable fluorogenic substrate PhiPhiLux-G1D2 (OncoImmunin Inc.; Gaithersburg, MD, USA), according to the manufacturer's instructions, and was imaged using fluorescence microscopy (Eclipse TE200; Nikon Instruments, Melville, NY, USA). (A) DMC not only decreased the number of FaDu cells in a dose-dependent manner, but also altered their morphology. (B) DMC increased the number of FaDu cells with condensed nuclei. (C) DMC increased the activity of caspase-3 in FaDu cells. Black arrow indicates the cells with altered morphology. White arrow indicates the cells with condensed chromatin.

Changes in the text: we have modified our text as advised (see Page 24, line 2-3) as below
→ Black arrow indicates the cells with altered morphology. White arrow indicates the cells with condensed chromatin.

3. Line 203: figure no should be 3C.

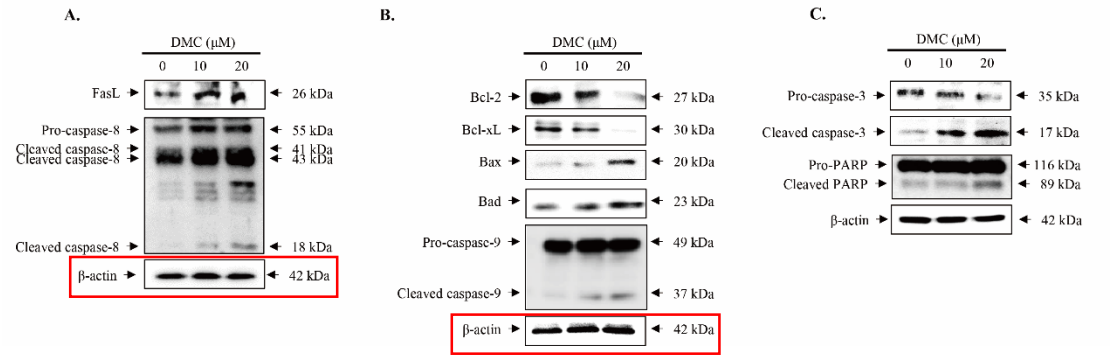
Reply: Thank you very much for your indication. It was revised as below.

Furthermore, DMC increased the number of FaDu cells with condensed nuclei, a representative characteristic of apoptotic cell death. Sequentially, the caspase-3/-7 activity assay showed that DMC increased the activity of caspase-3 in FaDu cells, as shown in Fig. 3C.

Changes in the text: we have modified our text as advised (see Page 10, line 7) as below
→ shown in Fig. 3C.

4. Fig 5A and B needs internal control.

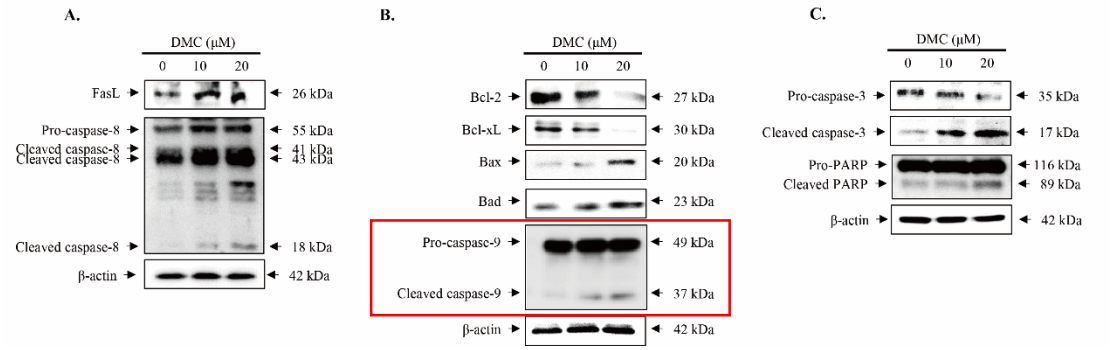
Reply: In accordance with reviewer's indication, western blot for β-actin were added in the Fig. 5A and 5B. Please see revised manuscript and below.



5. Fig 5B and C: for all Caspases, cleaved and pre- form should be in same blot like Fig 5A.

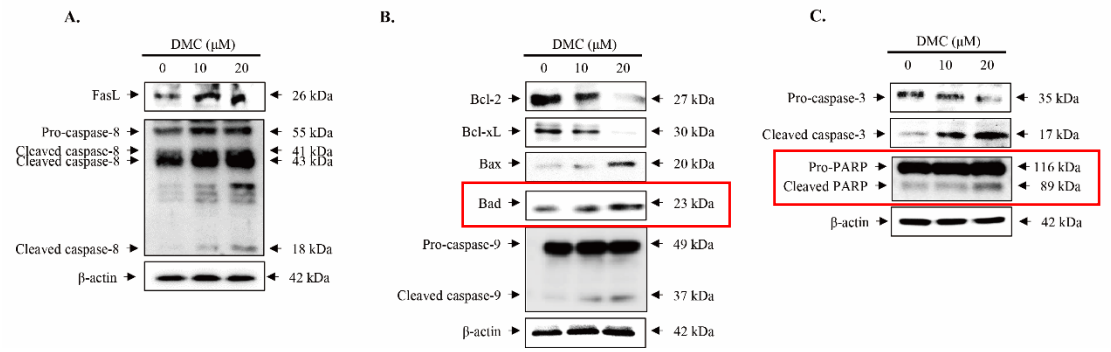
Reply: In accordance with reviewer's indication, the western blot for caspase-9 was performed again using caspase-9 antibody supplied from cell signaling (Cat. # 9508). To verify the expression of both pro- and cleaved caspase-3 on same blot, the western blot was performed using caspase-3 antibody (Cell signaling, Cat. # 14220) that can detect both pro- and cleaved

caspase-3. However, this antibody detected only pro-caspase-3. Hence, to detect the expression of cleaved capase-3, western blot was performed using cleaved caspase-3 specific antibody (Cell signaling, Cat #9664). Please understand it.



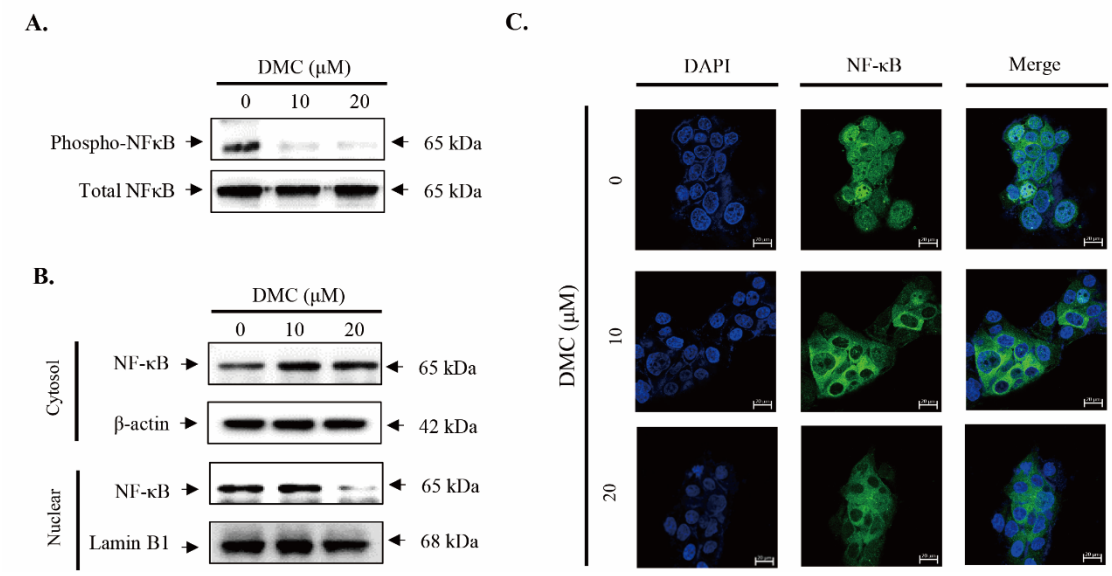
6. The expression image of Bad and PARP are not convincing due to unacceptable cropping of gel image; thus, the data should be reevaluated.

Reply: Thank you very much for your indication. It was revised as below.



7. Fig 6C: quality is very poor; nucleus is not prominent. Cells are of different sizes which is not like Fig 3. Where all the experiments were done at 24h then why exception in Fig 6C: 48h?

Reply: Thank very much for your indication. During the preparation of confocal image, we made a mistake. I apologize my mistake. It was revised as 24 h in the Figure 6C. In accordance with reviewer's indication, the translation of NF-κB in Figure 6C was repeated again to enhance the image. Please see revised manuscript and below. Thank you.



8. Authors should provide a schematic diagram showing mode of action of the drug.

Reply: According to reviewer's suggestion, the schematic diagram was added. Please see revised manuscript and below.

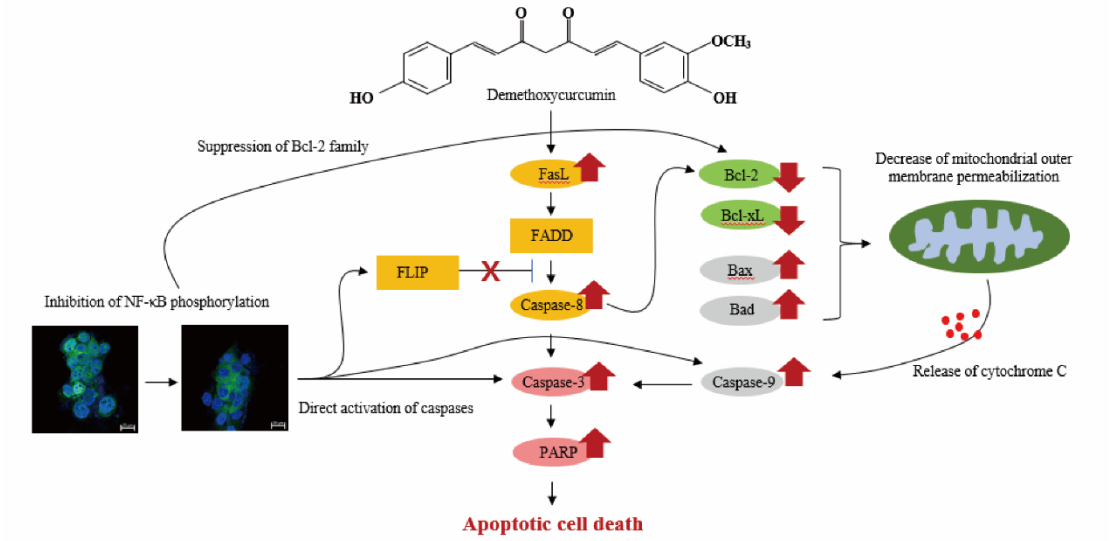


Figure 7 Schematic diagram of DMC-induced apoptosis in FaDu cells.

Changes in the text: we have added the figure legend for Figure 7 (see Page 16, line) as below

→ In conclusion, as shown in Figure 7, DMC-induced cell death was mediated through the caspase-dependent apoptosis pathway involved in the inhibition of NF-κB activation in FaDu cells.

Changes in the text: we have added the figure legend for Figure 7 (see Page 25, line 10) as below

→ Figure 7 Schematic diagram of DMC-induced apoptosis in FaDu cells.